The Role of Glycerol in the Osmotic Regulation of the Halophilic Alga *Dunaliella parva*

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ABSTRACT

Dunaliella parva, a green halophilic alga, was found to accumulate very large amounts of intracellular glycerol. Through measurements of the intracellular volume the internal concentration of glycerol was calculated and found to be around 2.1 M in cells cultured in 1.5 M NaCl. When the extracellular salt concentration of an algal suspension was increased or decreased, the intracellular glycerol varied accordingly, reaching its new osmotic equilibrium after about 90 minutes. Since no leakage of intracellular glycerol was observed above 0.6 M NaCl, these alterations in glycerol content are interpreted as due to metabolic formation and degradation of intracellular glycerol. The above results indicate the existence of a new type of algal osmoregulation, in which the osmotic balance depends on the synthesis or degradation of intracellular glycerol in response to the external salt concentration.

Algae tend to maintain an internal osmotic pressure somewhat above that of the medium. The green halophilic alga *Dunaliella* has an unusual requirement for high salt concentration for growth (7) and for high external osmotic pressure for photosynthetic oxygen evolution (2) and glycerol production (18). *Dunaliella* cells are often subjected to widely fluctuating salt concentrations and can tolerate a broad range of salt from low salinity to almost saturated NaCl solutions. The basis for this salt tolerance and the mechanism responsible for the osmotic regulation in *Dunaliella* have not been elucidated. Several reports suggest that the osmotic balance of *Dunaliella* is mediated by the existence of high salt concentrations inside the cells. Measurements of freezing point depression on cell sap of *Dunaliella salina* indicated that the internal osmotic concentration is higher than that due to the salt level of the medium (11). These authors concluded from studies of sodium uptake and exchange that the flagellates are highly permeable, especially to NaCl. Ginzburg (5) showed that the cytoplasmic membrane of *D. parva* is permeable to high molecular weight substances, such as inulin, and hence suggested that the cytoplasmic membrane is highly permeable. Electron microscopic observations showed that *D. salina* lacks a rigid cell wall and the cells are surrounded by only a cytoplasmic membrane (16). Since external osmotic changes induced reversible cell volume changes and exchange of water between the cells and the medium, these observations were interpreted as indicating a rapid exchange of salts through the cell membrane.

In contrast to the above observations direct measurement of the internal concentration of Na, K, and Cl in a halophilic strain of *Chlamydomonas* showed salt levels much lower than the external concentration, not exceeding 0.2 M NaCl when the cells were suspended at 1.7 M NaCl (14). Determination of the salt sensitivity of enzymic and photosynthetic reactions of cell-free preparations from *D. parva* (2) and *Dunaliella viridis* (7) showed inhibition rather than stimulation by NaCl concentrations far lower than those required for growth. The observations that glucose can replace NaCl for O₂ evolution in *D. parva* (2) and cannot be assimilated in *Dunaliella tertiolecta* (9) indicate that the cells are impermeable to glucose. Thus, these observations indicate that the inside of these cells maintains a considerably lower salt concentration than that present in the surrounding medium (2, 7, 14).

Photosynthetic production of glycerol in response to the salt concentration and osmotic pressure of the medium was previously described in *D. tertiolecta* (4, 18). The purpose of this communication is to demonstrate that free glycerol is produced and accumulated in *D. parva* cells, to the final concentration needed to balance the external salt concentration. Osmoregulation in *Dunaliella* will be shown to depend on the formation and degradation of intracellular glycerol.

MATERIALS AND METHODS

Algae. *Dunaliella parva* was cultured as previously described or, where indicated, with supply of 5% CO₂ (12). *Euglena gracilis* cells were grown in Hutner medium (6). *Phormidium luridum* cells were grown, and protoplasts were prepared as previously described (3).

Algal Extract Preparation. Extract of *D. parva* was prepared by diluting the concentrated algae (suspended in 1.5 M NaCl, 5 mM tris, pH 7.5) in 5 mM tris, pH 7.5, at 0 C, resulting in a final NaCl concentration of 25 mM. After 10 min, the fragments obtained (2) were removed by centrifugation at 12,000g for 10 min. Extract of *E. gracilis* was prepared by sonication of the cells for 20 sec at 4a in a Branson sonifier equipped with a microtip at 0 C. The broken cells were removed by centrifugation. Extract of *P. luridum* was obtained by lysis of the protoplasts in 25 mM Tricine, pH 7.5, followed by centrifugation. The supernatants obtained from these algae were deproteinized at 2% trichloroacetic acid, neutralized with NaHCO₃, and analyzed for glycerol by the enzymic method described below.

Enzymic Determination of Glycerol. The glycerol content in the algal cells was determined by a specific enzymic oxidation of glycerolphosphate by NAD to dihydroxyacetone phosphate and NADH (19). A sample of the algal extract was added to the reaction mixture containing: 0.2 M glycine, pH 9.8; 7.5 mM NaCl; 2 mM MgCl₂; 1.0 M hydrazine; 0.75 mM NAD; 15 µg of α-glycerophosphate dehydrogenase (Sigma); and 1 mM ATP. The reaction was started by the addition of 7

μg of glycerokinase (Sigma). The final volume was 3 ml. The reduction of NAD was followed in a Cary model 16 spectrophotometer, at 340 nm.

Measurements of Glycerol Content in *D. parva*. The growth culture of *D. parva* was concentrated by centrifugation at 1,500*g* for 10 min at room temperature, washed twice in 1.5 M NaCl, 5 mM buffer phosphate, pH 7.5 (tris or Tricine interfere in the chemical assay of glycerol). Equal samples of the concentrated algae, each containing about 100 μg of chlorophyll, were diluted in a series of tubes containing 16 mM buffer phosphate, pH 7.5; 2 mM MgCl$_2$; and the desired NaCl concentration to a final volume of 1 ml. All tubes were placed in a water bath at 25 C. Two tubes of zero time (containing 3% trichloroacetic acid) and two of blank tubes (without algae) were run in each experiment. Where indicated light was supplied by lamps of about 45,000 lux. The reaction was stopped by addition of 0.1 ml of 30% trichloroacetic acid to each tube. The same final NaCl concentration in the whole series was maintained by adding NaCl and water. Clear supernatant was obtained by centrifugation at 1,000*g* for 10 min at 0C. In the glycerol leakage experiment, cells and broken cells were removed first by centrifugation and only later trichloroacetic acid, NaCl, and water were added to the supernatant and the tubes were centrifuged again. Then 0.5 ml of the deproteinized clear supernatant was transferred to new tubes containing 1.5 ml of water, and glycerol was assayed by the chemical determination, as described below.

Chemical Determination of Glycerol. To a series of tubes containing 1.5 ml of water, 0.5 ml of a deproteinized solution containing 0.1 to 1.5 μmoles of glycerol was added, and glycerol was assayed after Lambert and Neish (10). Then 0.1 ml of 10 N H$_2$SO$_4$ and 0.5 ml of 0.1 M sodium periodate were added with mixing to each tube. Five minutes after the addition of periodate, 0.4 ml of 1 M sodium arsenite was added to each tube of the series. About 5 min after addition of arsenite, 0.1 to 0.5 ml from each tube was pipetted into a new tube followed by the addition of 10 ml of 6.3 mM chromotrophic acid reagent (sodium 1,8-dihydroxynaphthalene-3,6-disulfonate) (10) with mixing. The tubes were placed for 30 min in a boiling water bath and allowed to cool, and the absorbance was read at 570 nm in a spectrophotometer. A blank without algae and a calibration curve of pure glycerol were run with each set.

Pellet sorbitol space and water space were measured following the method of Rottenberg *et al.* (15). Dry weight and pellet water were measured also by weight of packed cells in small glass tubes before and after heating at 80 C. Chlorophyll was assayed after Arnon (1).

RESULTS

Glycerol Concentration in *D. parva*. The glycerol content of *D. parva* was determined enzymically to be around 60 μmoles/mg of chlorophyll. Undetectable levels of glycerol were found in *E. gracilis* and *P. luridium*, by the same assay procedure. Table I shows that under standard cultural conditions when NaCl concentration of the medium is 1.5 M the glycerol content inside *D. parva* is around 50 μmoles of glycerol per mg of chlorophyll, and more than doubled when the algae were cultured with 5% CO$_2$. The actual intracellular concentration of glycerol was determined using the pellet water and sorbitol space (nonosmotic space). As illustrated in Table I, the concentration of glycerol was around 2.1 M for both algal cultures. Since 2.1 M glycerol is osmotically equivalent to 1.4 M NaCl, it is clear that glycerol serves as the major osmotic component in the halophilic alga. If it is assumed that the amount of chlorophyll in the cells of both cultures is similar and as shown, the concentration of glycerol is the same, then

Table I. *Determination of Intracellular Glycerol Concentration in D. parva*

Culture Conditions	Extracellular NaCl Concn	Intracellular Glycerol Content	Total Pellet Water	Pellet Sorbitol Space	Cell Water	Dry wt	Cell Water	Intracellular Glycerol Concn
	M	*μmoles/mg chl*	*μl H₂O/mg chl*		*μl H₂O/mg chl*	*mg/mg chl*	*μl H₂O/mg dry wt*	*M*
Control	1.5	60	42.81	0.27	31.25	23.54	1.33	1.92
	1.5	50	32.0	0.27	23.36	...	...	2.15
+ 5% CO₂	1.5	131	85.29	0.27	62.26	42.33	1.47	2.10

Total pellet water and dry wt content were determined from cells suspended in 1.5 M NaCl, 10 mM tris-Cl, pH 7.2. Pellet sorbitol space and cell water were determined in a basic reaction mixture containing 1.5 M NaCl; 10 mM tris-Cl, pH 7.2; 5 mM sorbitol; *D. parva* cells containing 400 μg of chl/ml, 2 μc/ml ^{3}H$_2$O, and 2 μc/ml ^{14}C-sorbitol. Glycerol was determined by the enzymic method. For other details see "Materials and Methods."
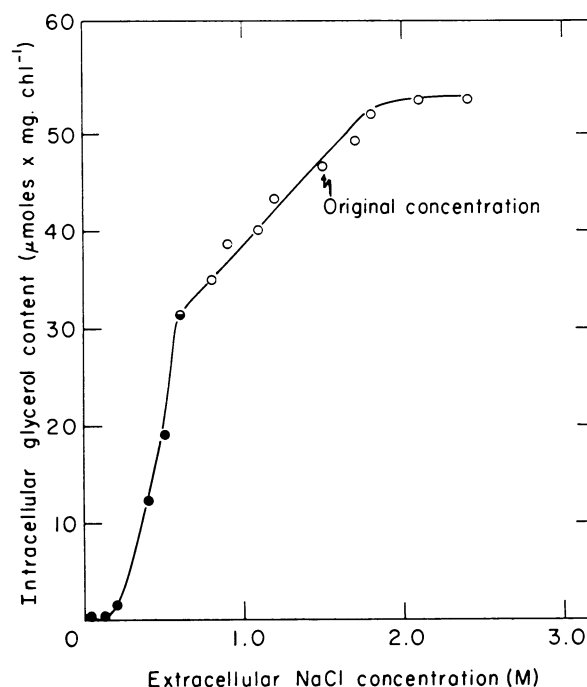

FIG. 1. Effect of the NaCl concentration of the medium on the intracellular content of glycerol in *D. parva*. The reaction mixture contained: 5 mM phosphate pH 7.5, 2 mM MgCl$_2$, the indicated concentration of NaCl and *D. parva* cells containing 100 μg of chlorophyll in a total volume of 1 ml. After 90 min the reaction was stopped by addition of 0.1 ml of 30% trichloroacetic acid. ○: Intracellular glycerol content; ●: intracellular glycerol content calculated by substracting the content of glycerol excreted into the medium from total glycerol content. For further details see text and "Materials and Methods."

the differences in glycerol, water, and dry weight contents calculated on chlorophyll basis reflect a larger cell volume in the algae grown with CO$_2$.

Effect of NaCl Concentration on the Glycerol Content in *Dunaliella* Cells. Figure 1 illustrates the marked effect of NaCl concentration upon the glycerol content of the cells. Upon addition of NaCl above the salt level of the culture medium an increase in glycerol is observed. When the osmolarity of the medium is decreased, glycerol content decreases. A linear relation is held over a broad range of salt concentration from 0.6 to 2.1 M. Below 0.6 M NaCl, glycerol leaks or is excreted into the medium in gradually increasing amounts (Fig. 2). To learn

the intracellular content of glycerol below 0.6 M NaCl, we subtracted the amount of glycerol excreted into the medium at any point from the total glycerol content. These calculated intracellular glycerol contents are plotted as the closed circles of Figure 1. Since at NaCl concentrations above 0.6 M glycerol is not excreted into the medium, the salt-induced glycerol changes must depend on metabolic formation and degradation of glycerol. As illustrated in Figure 2 and as already described (2) at low osmotic pressures, the cells burst, the degradation of glycerol stops, and the amount of glycerol that is released to
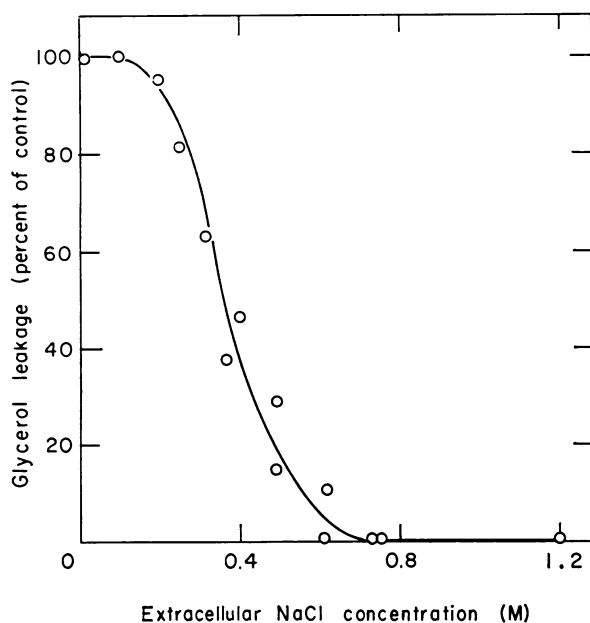

FIG. 2. Leakage of intracellular glycerol into the medium as function of the external NaCl concentration. The reaction mixture was the same as described in Figure 1. Ninety min after dilution, the cells and broken cells were removed, and glycerol content was determined in the medium. 100% refers to 60 μmoles of glycerol/mg chl. For other details see "Materials and Methods."
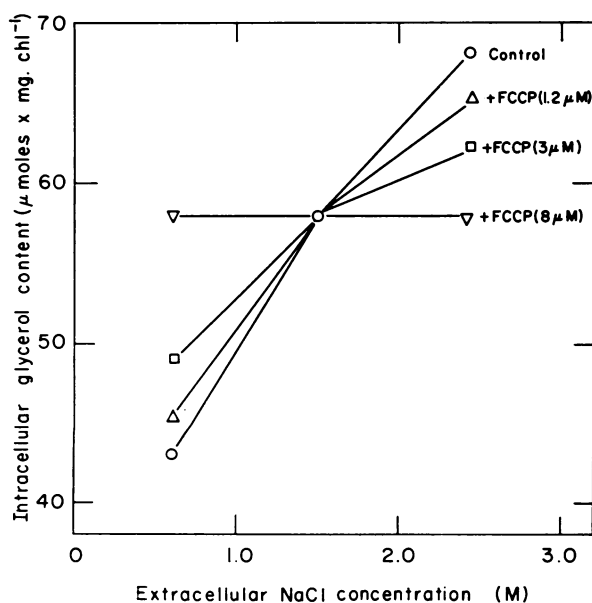

FIG. 3. Effect of FCCP on the changes in intracellular glycerol content induced by extracellular NaCl in D. parva. The reaction mixture and time were the same as described in Figure 1, except for the addition of FCCP at the indicated concentrations.
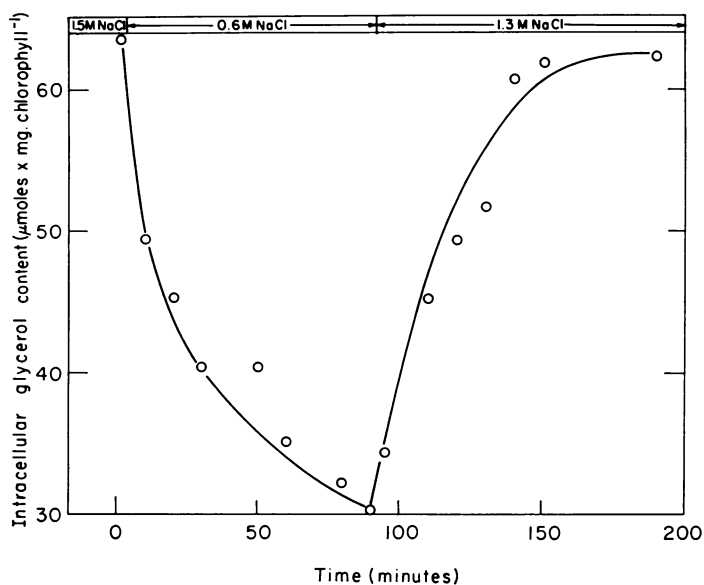

FIG. 4. Kinetics of degradation and synthesis of glycerol in D. parva in response to changes in the salt concentration of the medium. Concentrated algae suspended in 1.5 M NaCl, 5 mM phosphate, and 2 mM MgCl, pH 7.5, were diluted to obtain a final concentration of 0.6 M NaCl, 5 mM phosphate, and 2 mM MgCl₂, pH 7.5. Ninety min after dilution, solid NaCl was added to a final concentration of 1.3 M. For further details see "Materials and Methods."

the medium equals the intracellular glycerol content of algae suspended at 1.5 M NaCl.

Figure 3 shows the effect of the uncoupler FCCP[1] on the NaCl-induced glycerol changes in D. parva. Both formation and degradation of glycerol were inhibited completely by 8 μM FCCP, and the glycerol level remained constant over the whole range of NaCl concentration, implying an essential requirement for ATP in the metabolism of glycerol.

The kinetics of the salt-induced changes in internal glycerol content are shown in Figure 4. The left side of the figure describes the decrease in glycerol content following dilution of the medium from 1.5 to 0.6 M NaCl, and the right side describes the increase in glycerol content upon addition of NaCl to the medium. In both cases the algae reach their new osmotic equilibrium after about 90 min. This kinetic behavior was observed in the light or in the dark.

DISCUSSION

The accumulation of nonreducing low molecular weight carbohydrates appears to be a common feature of algal metabolism (13). Dunaliella represents a special case of a photosynthetic production of free glycerol in response to the osmotic pressure of the medium (4, 18). Our data indicate that D. parva produces and accumulates large amounts of glycerol within the cell. Based on measurements of water content in algae suspended at 1.5 M NaCl an internal concentration of 2.1 M glycerol was calculated. Our observation that glycerol is not excreted into the medium suggests that the major function of glycerol is to maintain the osmotic balance in D. parva. Thus, glycerol formation and degradation should be regarded as an osmoregulatory mechanism necessary to maintain a suitable osmotic pressure within the cells.

Previous investigators (16) have concluded, on the basis of

[1] Abbreviation: FCCP: carbonyl cyanide p-trifluoromethoxy phenylhydrazone.

rapid exchange of water and cell volume changes of *D. salina*, that the reversible cell volume changes are mediated by fluxes of NaCl through the cytoplasmic membrane. Our data show that these results were due to the formation or degradation of internal glycerol following the osmotic changes of the medium. As a consequence, an equilibrium between internal and external osmotic conditions is established in a sufficiently short period of time which is required for glycerol to reach its new level.

The results described demonstrate that the formation and degradation of glycerol are not light-dependent. However, since it has been shown (4, 17, 18) that glycerol is a photosynthetic product of *Dunaliella*, it is reasonable to assume that two different metabolic pathways may be responsible for glycerol formation: one using a photosynthetic product and the other via the metabolic degradation of starch, the storage product in *Dunaliella*. A closely related mechanism of isofloridoside formation induced by increase of external osmotic pressure has been described in the fresh water alga *Ochromonas* (8), but the effective range of external osmotic pressure is much lower than in *Dunaliella*.

In analogy with halophilic bacteria the halophilic alga *Dunaliella* requires high salt concentrations for optimal growth and photosynthetic activities (4, 7). But in contrast to the salt tolerance of enzymes extracted from halophilic bacteria, enzymic and photosynthetic activities of *Dunaliella* were inhibited by NaCl concentrations far lower than those required for growth (2, 7). Thus, the enzymes of *Dunaliella* cells, which are salt-sensitive, would be expected to be resistant to inactivation by glycerol or may even require substantial concentrations of glycerol for full enzymic activity. The behavior of *D. parva* enzymes under high glycerol concentrations and the pathway of glycerol formation are presently under investigation, but preliminary results already obtained indicate that the above prediction holds true, at least in some cases.

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